REMARKS/ARGUMENT

Applicants acknowledge receipt of the Office Action dated March 4, 2004 in which certain claims stand rejected under 35 U.S.C. § 112, first paragraph; certain claims are rejected under 35 U.S.C. § 112, second paragraph; and certain claims are rejected under 35 U.S.C. § 102(b) as anticipated by *Russell.* Claims 23, 24, 26 and 42 are allowed. Claims 1-21, 25, 27-41, 43-44 and 47-66 have been canceled without prejudice, and claims 45, 46, 67 and 68 have been amended. Claims 23, 24, 26, 42, 45, 46, 67 and 68 are pending.

Rejection of Claims Under 35 U.S.C. § 112, First Paragraph - Written Description

Claims 45, 46, 67 and 68 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking adequate written description. In the Office Action it is suggested that fragments/variants of SEQ ID NO: 2 or 4 do not meet the guidelines on written description. To the contrary, the specification expressly describes peptides that are at least 60% or at least 90% identical to the CSP peptides (SEQ ID NO. 2 or 4). See paragraphs 38 and 54 of the specification, for example. Amended claims 45, 46, 67 and 68 conform with at least Example 14 of the Written Description Guidelines published at the internet address http://www.uspto.gov/web/menu/written.pdf.

Rejection of Claims Under 35 U.S.C. § 112, First Paragraph - Enablement

Claims 45, 46, 67 and 68 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement. Applicants respectfully traverse and submit that the specification indicates in paragraphs 37-38 how such 60% or 90% identical peptides may be prepared. Moreover, it is generally known in this field how to alter DNA sequences by addition, deletion and substitution of nucleic acids. In the present case, it is clear that a reasonable and finite number of DNA sequences are contemplated in amended claims 45, 46, 67 and 68. Given Applicants' disclosure, only routine effort would have been required by one of skill in the art to produce the 60% or 90% identical sequences, and to test those sequences for CSP activity, as described in the specification. See paragraphs 36, 78 and 79 of the specification, for example. Attached in support hereof is the *Declaration of Dr. Dennis Cvitkovitch.*

Rejection of Claims Under 35 U.S.C. § 102(b)

Claims 45, 46, 67 and 68 stand rejected under 35 U.S.C. § 102(b) as being anticipated by U.S. Patent No. 4,521,513 (*Russell*). It is suggested in the Office Action that the antigenic protein C described by *Russell* reads on the instantly claimed polypeptide fragments/variants of SEQ ID NO. 2

or 4. This allegation is rebutted by the *Declaration of Dr. Dennis Cvitkovitch*, and the attachments thereto.

Applicants deny the allegation on page 10, lines 16-19 of the Office Action that "Applicant stated on October 15, 2003 that the antigen disclosed by Russell is naturally competent." Such allegation misstates Applicants actual statement in the *Amendment and Response to Office Action Dated December 13, 2002* (on page 8),

> *Although, the strain Ingbritt used to isolate antigen C is naturally competent, the growth conditions described by Russel are not competence inducing, therefore very little CSP should be produced prior to his purification process.*

The *Declaration of Dr. Dennis Cvitkovitch* discusses the protein C of *Russell* and refutes the Office Action's erroneous assertion that the protein C of *Russell* necessarily has CSP activity.

Conclusion

Applicants may have at times referred to claim limitations in shorthand fashion, or may have focused on a particular claim element. This discussion should not be interpreted to mean that the other limitations can be ignored or dismissed. The claims must be viewed as a whole, and each limitation of the claims must be considered when determining the patentability of the claims. Moreover, it should be understood that there may be other distinctions between the claims and the prior art, which have yet to be raised, but which may be raised in the future. In the interest of advancing issuance of a patent, Applicants have chosen to cancel certain claims. Such cancellation is not an admission as to the correctness of any rejection.

Consideration of the foregoing amendments and remarks, reconsideration of the application and withdrawal of the rejections and objections is respectfully requested by Applicants. No new matter is introduced by way of the amendments. If any item in the Office Action has been overlooked or is deemed to be incompletely addressed, Applicants respectfully request the opportunity to respond. Applicants believe that no extension of time is necessary for this paper to be deemed timely filed. If a petition for extension of time is necessary in order for this paper to be deemed timely filed, please consider this a petition therefor. If any fee is due as a result of the filing of this paper please

appropriately charge such fee to Deposit Account Number 03-2769 of Conley Rose, P.C., Houston, Texas.

Respectfully submitted,

Carol G. Mintz

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AGENT FOR APPLICANT

CONFIDENTIAL AND PRIVILEGED
ATTORNEY CLIENT COMMUNICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT:	Dennis Cvitkovitch et al.	§	**GROUP ART UNIT:** 1645
SERIAL NO.:	09/833,017	§	
FILED:	April 10, 2001	§	**EXAMINER:** Padmavathi Baskar
FOR:	Signal Peptides, Nucleic Acid Molecules and Methods for Treatment of Caries	§	

Atty. Dkt. No.: 1889-00401
Date: June 4, 2004

DECLARATION OF DR. DENNIS CVITKOVITCH

Commissioner for Patents
Washington, D.C. 20231

Sir:

PURPOSE OF DECLARATION

This declaration is to rebut the enablement and written description rejections and to establish the deficiency of at least the following reference as prior art against the above-identified U.S. patent application:

United States patent US 4,521,513 issued to Russell (*'Russell'*).

STATEMENT OF FACTS

1. I, Dr. Dennis Cvitkovitch, have a Doctorate Degree, and I am currently a tenured Associate Professor in the Faculty of Dentistry and in the Institute of Biomedical and Biomechanical Engineering (IBBME) at the University of Toronto. I also hold a tier 2 Canada

Research Chair. I have been studying the basic physiology and pathogenic properties of the dental bacterium Streptococcus mutans since 1986. I have Authored and co-Authored 25 manuscripts in peer-reviewed journals that Research focused on the study or this bacterium I have also authored 4 review articles that I was invited to write. I have been invited to many international conferences including the American Society for Microbiology's conference on Streptococcal genetics in 2002 and the Biofilm meeting in 2003. Further evidence supporting my recognition as an expert in the field of dental caries and *S. mutans* is evident by the more than 2 million dollars in grants that I have received from the US National Institutes of Health (NIH) and the Canadian Institutes of Health Research (CIHR). I am currently the only Canadian to receive funding from both of these prestigious agencies to study oral bacteria.

2. I am familiar with the level of technical knowledge possessed by those who work in the field of making and testing peptides for competence signal peptide activity ('CSP activity') and I am of the opinion that the standard or usual level of skill of such individuals is approximately a Doctorate degree and at least 5 years of experience in the field.

3. One of ordinary skill in the art of making and testing peptides for competence signal peptide activity ('CSP activity') *i.e.* one having the level of knowledge described in item 2. above, at the time the invention was made, would find in the specification adequate teaching of how to identify the claimed 60% or 90% identical sequences to SEQ ID NO:2 or SEQ ID NO:4. Given the present disclosure, a person skilled in this technology would readily appreciate that a variety of homologous peptide sequences can be used for the claimed activity, namely CSP activity and inhibition of CSP activity. For example, an assay of genetic competence is described on page 22, starting at line 5 of the application, and an assay of acid resistance tolerance is described on page 23, starting at line 12 of the application. The specification therefore, gives instructions which are sufficient to enable a skilled person to use a range of homologous peptides without undue experimentation or further invention. A skilled person would understand the limitations of the claims as written and would readily recognize whether a given sequence falls within or outside the scope of the claim.

4. One of ordinary skill in the art of making and testing peptides for competence signal peptide activity, when reading *Russell* would not accept as true that the protein antigen C disclosed in *Russell* necessarily and inevitably, comprises SEQ ID NO:2 or SEQ ID NO:4 of the current invention. Specifically, one of ordinary skill in the art would know that there is only one location for the CSP sequence in the *S.mutans* genome and it is in an open reading frame consisting of the comC gene, which encodes the CSP precursor protein, ComC. There is no other sequence in the *S. mutans* genome, that, even processed post translationally, can yield CSP. One of skill in the art, upon carrying out a routine search on the CSP sequence (SEQ ID NO:4), and calculating typical parameters for that sequence, using well-known publicly available resources, would conclude that the Antigen C of the *Russell* publication does not inherently (or otherwise) comprise the CSP (SEQ ID NO;4) or ComC (SEQ ID NO:2) polypeptides of the claimed invention.

5. Submitted together with this Declaration are copies of *S.mutans* genome database analysis which clearly demonstrates that no other protein, including Antigen C, may contain the CSP sequence.

6. By way of personal communication with Dr. R. Russell on September 11, 2003, the inventor of the current invention was informed that Antigen C is likely to be the glucan-binding protein C ('GbpC'). GbpC is a 70kDa protein and has an estimated pI of 4.96. The CSP of the current invention is a 21 amino acid extracellular communications molecule with an estimated pI of 12.28. GbpC also contains an LPXTG amino acid motif, which is noticeably absent form SEQ ID NO:2 and SEQ ID NO:4.

7. Submitted together with this Declaration are copies of the computational analysis (database search, pI, MW, hydrophobicity profiles, secondary structure predictions, charge distribution analysis, pairwise alignment, protein composition analysis and stability predictions) performed on GbpC and CSP and copies of the following two papers on GbpC:

(1) Cloning and Sequence Analysis of the *gbpC* Gene Encoding a Novel Glucan-Binding Protein of *Streptococcus mutans*; and

(2) Attenuation of Glucan-binding Protein C Reduces the Cariogenicity of *Streptococcus mutans*: Analysis of Strains Isolated from Human Blood.

Taken separately or together, these documents clearly demonstrate that gbpC is not the CSP of the current invention.

TIME OF PRESENTATION OF THE DECLARATION

This Declaration is submitted with the Response to the Office Action dated March 4, 2004 and is for the purpose of overcoming one or more ground of rejection or requirement made in that Office Action

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE

Full name: Dennis Gerard Cvitkovitch

Signature:

Date: June 2/04 Country of Citizenship: CANADA

Residence Address: 1338 Odessa Cres, Oakville, ON CANADA L6H 1R8

Attachments to Declaration of Dr. Dennis Cvitkovitch

(1) Computational Differences between GbpC and CSP, NCBI database (http://www.ncbi.nlm.nih.gov/) search results as of September 11, 2003 – (10 pages)

(2) BlastP Analysis of the CSP peptide against the whole S. Mutans UA159 Protein Database, posted date June 2, 2004 – (2 pages)

(3) Yutaka Sato et al., Cloning and Sequence Analysis of the gbpC Gene Encoding a Novel Glucan-Binding Protein of Streptococcus mutans, INFECTION AND IMMUNITY, Feb. 1997, p. 668-675

(4) K. Nakano et al., Attenuation of Glucan-binding Protein C Reduces the Cariogenicity of Streptococcus mutans: Analysis of Strains Isolated from Human Blood, J DENT RES, 2002, 81(6): 376-379

Computational Differences between GbpC and CSP

The first section of the comparison is based on NCBI database (http://www.ncbi.nlm.nih.gov/) search results as of September 11, 2003. The following will show the differences between the CSP protein and the antigen C database records. Search for CSP was "ComC mutans" under proteins. Search for GbpC was "GbpC mutans" under proteins.

GbpC protein sequence:

>gi|24379801|ref|NP_721756.1| glucan-binding protein C, GbpC [Streptococcus mutans UA159]
MKSKTAKITLLSSLALAAFGATNVFADEASTQLNSDTVAAPTADTQASEPAATEKEQSPVVAVVESHTQG
NTTTTTSQVTSKELEDAKANANQEGLEVTETEAQKQPSVEAADADNKAQAQTINTAVADYQKAKAEFPQK
QEQYNKDFEKYQSDVKEYEAQKAAYEQYKKEVAQGLASGRVEKAQGLVFINEPEAKLSIEGVNQYLTKEA
RQKHATEDILQQYNTDNYTASDFTQANPYDPKEDTWFKMKVGDQISVTYDNIVNSKYNDKKISKVKINYT
LNSSTNNEGSALVNLFHDPTKTIFIGAQTSNAGRNDKISVTMQIIFYDENGNEIDLSGNNAIMSLSSLNH
WTTKYGDHVEKVNLGDNEFVKIPGSSVDLHGNEIYSAKDNQYKANGATFNGDGADGWDAVNADGTPRAAT
AYYGAGAMTYKGEPFTFTVGGNDQNLPTTIWFATNSAVAVPKDPGAKPTPPEKPELKKPTVTWHKNLVVE
TKTEEVPPVTPPTTPDEPTPEKPKTPEDPQSPVVAKSVSFRTARKGEMRVRERDYQPTLPHAGAAKQNGL
ATLGAISTAFAAATLIAARKKEN

Competence Stimulating Protein (CSP) Sequence:

>gi|24380265|ref|NP_722220.1| competence stimulating peptide, precursor [Streptococcus mutans UA159]
MKKTLSLKNDFKEIKTDELEIIIGGSGSLSTFFRLFNRSFTQALGK

The CSP protein is then cleaved to form the following peptide sequence:
SGSLSTFFRLFNRSFTQALGK
Reference: J Bacteriol. 2001 Feb;183(3):897-908. (Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG).

Strains from which the respective proteins were isolated/gene location/gene name .

CSP

Organism = Streptococcus mutans, strain="UA159".
Gene location: 1795008..1795148.
Gene name: ComC (Competence C) -> CSP

Glucan-binding protein C

Organism = Streptococcus mutans, strain="UA159".
Gene location: 1324324..1326075.
Gene name: GbpC

This section will deal with the various physical properties of both proteins as predicted by the following programs.

Physical Properties Prediction tools

MacVector 7.2 (local program)
ProtParam (http://us.expasy.org/tools/protparam.html)
SAPS - Statistical Analysis of Protein Sequences (http://www.ch.embnet.org/software/SAPS_form.html)

MacVector 7.2

CSP pI (iso electric point) and MW (molecular weight)

Calculated Molecular Weight = 2364.52
Estimated pI = 12.48
Amino Acid Composition:

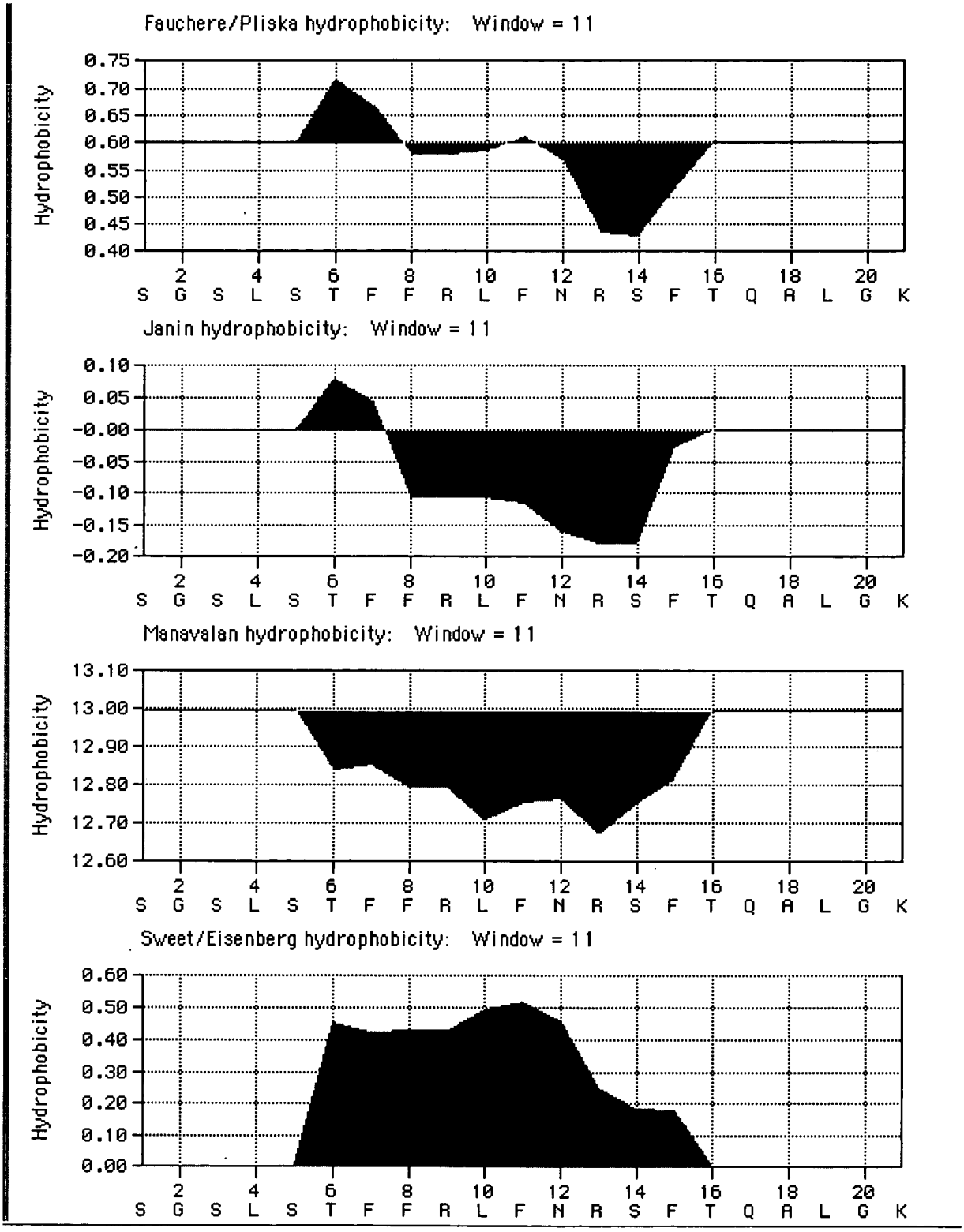
Non-polar:	No.	Percent
A	1	4.76
V	0	0.00
L	3	14.29
I	0	0.00
P	0	0.00
M	0	0.00
F	4	19.05
W	0	0.00
Polar:	**No.**	**Percent**
G	2	9.52
S	4	19.05
T	2	9.52
C	0	0.00
Y	0	0.00
N	1	4.76
Q	1	4.76
Acidic:	**No.**	**Percent**
D	0	0.00
E	0	0.00
Basic:	**No.**	**Percent**
K	1	4.76
R	2	9.52
H	0	0.00

GbpC pI (iso electric point) and MW (molecular weight)

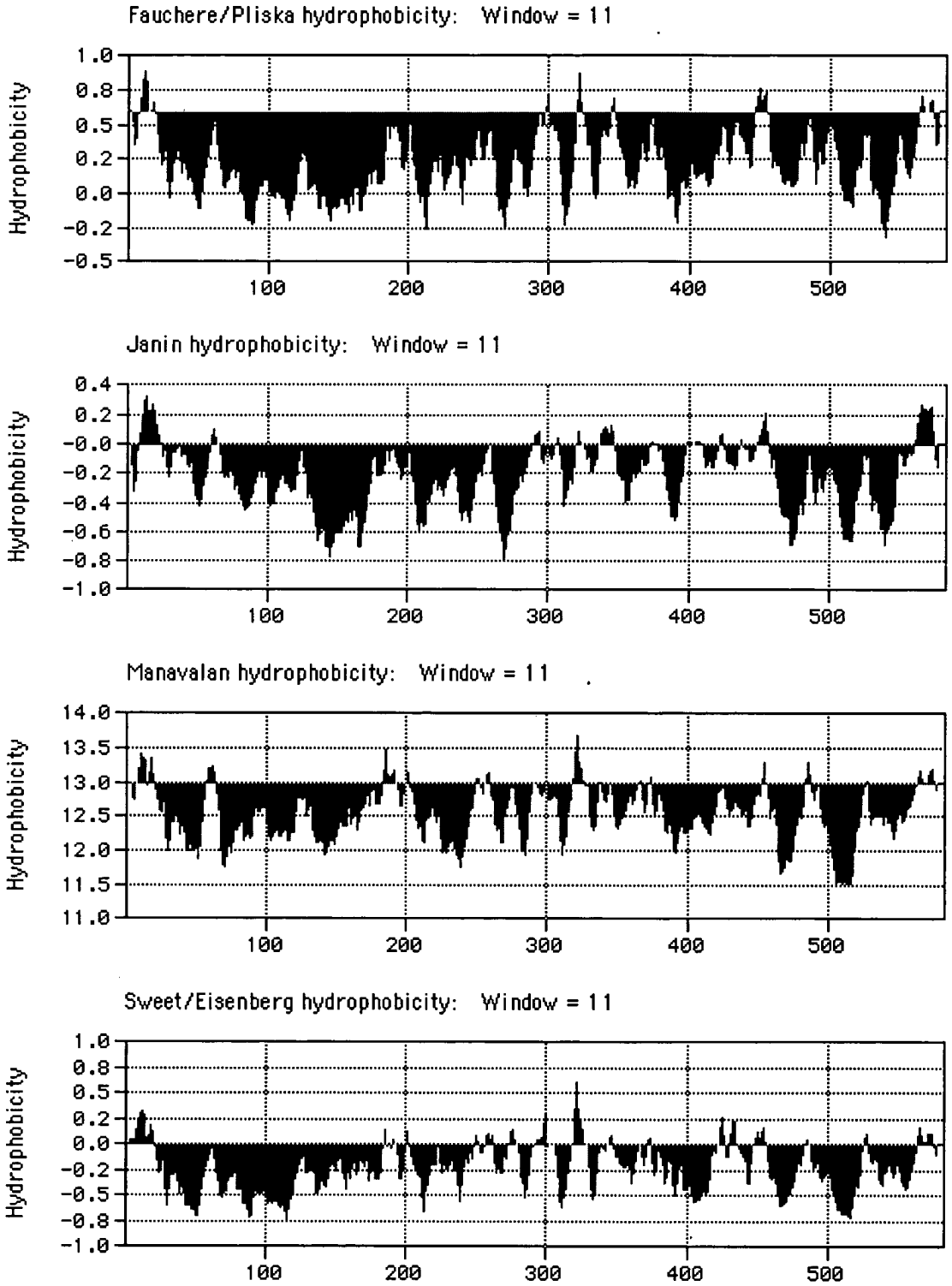
Calculated Molecular Weight = 63344.64
Estimated pI = 4.96
Amino Acid Composition:

Non-polar:	No.	Percent
A	70	12.01
V	38	6.52
L	27	4.63
I	21	3.60
P	33	5.66
M	6	1.03
F	17	2.92
W	5	0.86
Polar:	**No.**	**Percent**
G	32	5.49
S	35	6.00
T	58	9.95
C	0	0.00
Y	21	3.60
N	41	7.03
Q	32	5.49
Acidic:	**No.**	**Percent**
D	34	5.83
E	44	7.55
Basic:	**No.**	**Percent**
K	51	8.75
R	10	1.72
H	8	1.37

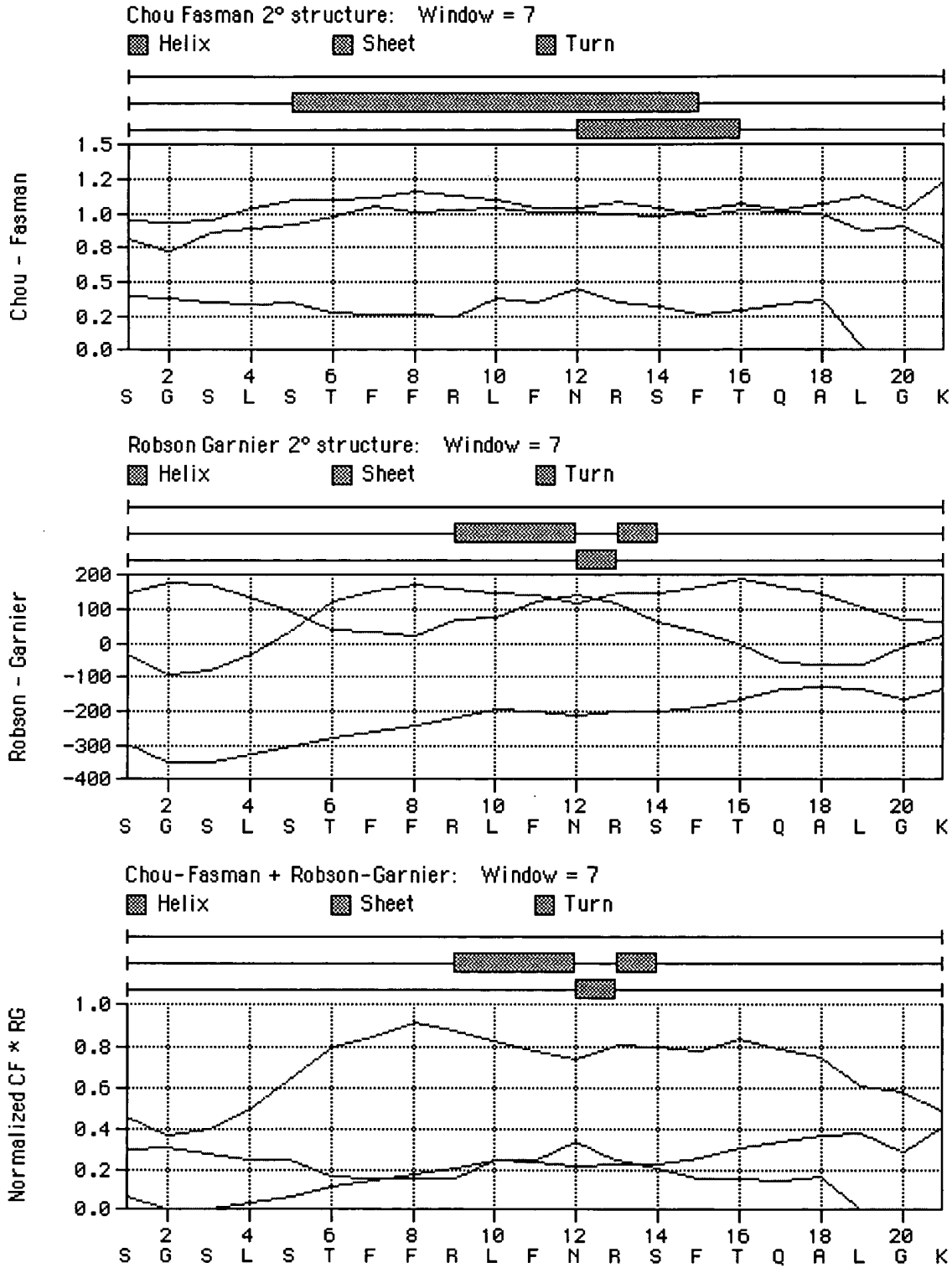
Hydrophobicity profile of CSP



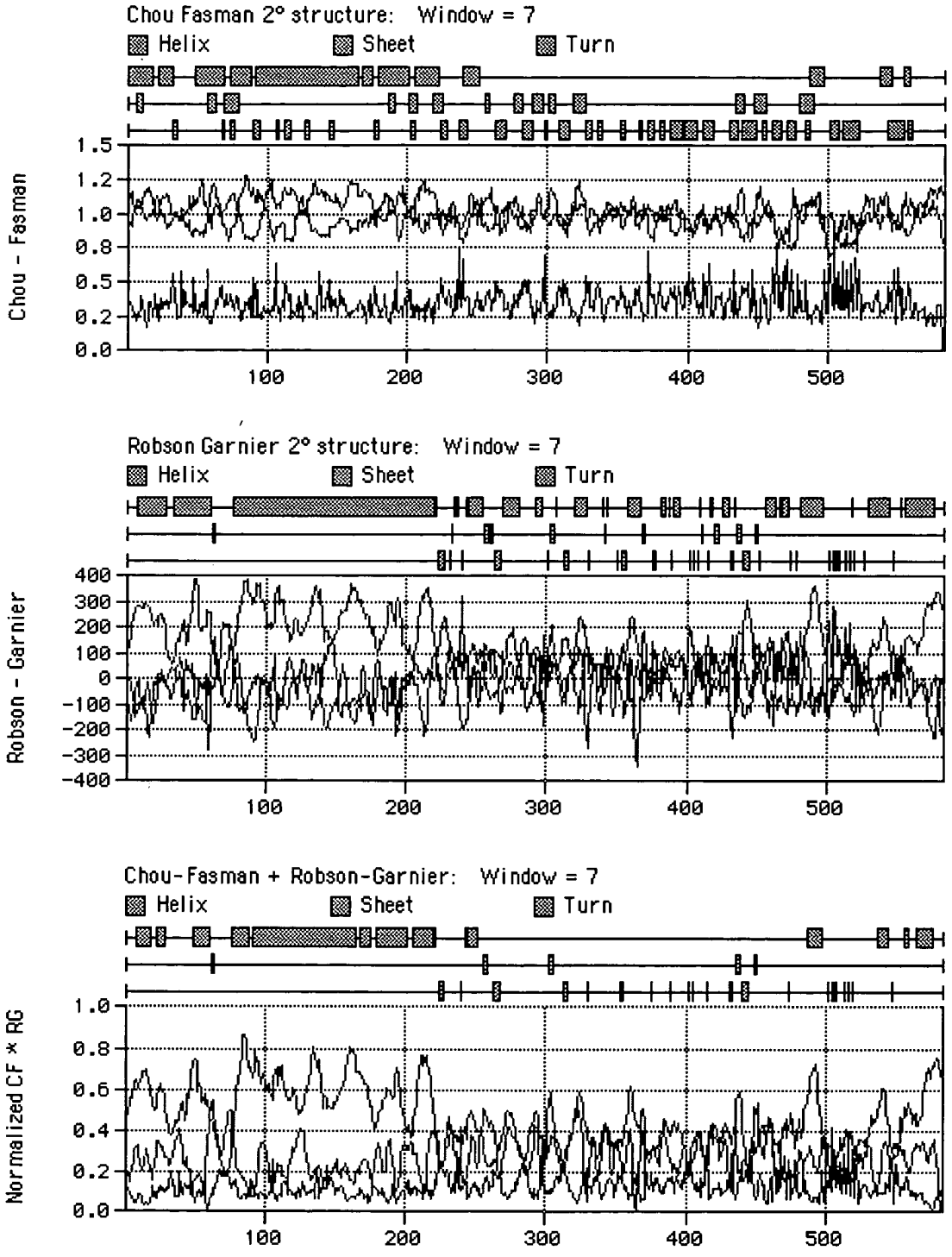
Hydrophobicity profile of GbpC



Secondary structure of CSP



Secondary structure prediction of GbpC



ProtParam

Results for the CSP protein

Number of amino acids: 21 **Molecular weight:** 2364.6 **Theoretical pI:** 12.01

Amino acid composition:

Ala (A)	1	4.8%
Arg (R)	2	9.5%
Asn (N)	1	4.8%
Asp (D)	0	0.0%
Cys (C)	0	0.0%
Gln (Q)	1	4.8%
Glu (E)	0	0.0%
Gly (G)	2	9.5%
His (H)	0	0.0%
Ile (I)	0	0.0%
Leu (L)	3	14.3%
Lys (K)	1	4.8%
Met (M)	0	0.0%
Phe (F)	4	19.0%
Pro (P)	0	0.0%
Ser (S)	4	19.0%
Thr (T)	2	9.5%
Trp (W)	0	0.0%
Tyr (Y)	0	0.0%
Val (V)	0	0.0%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 0

Total number of positively charged residues (Arg + Lys): 3

Atomic composition:

Carbon	C	108
Hydrogen	H	166
Nitrogen	N	30
Oxygen	O	30
Sulfur	S	0

Formula: C108 H166 N30 O30

Total number of atoms: 334

Extinction coefficients: As there are no Trp, Tyr or Cys in the region considered, your protein should not be visible by UV spectrophotometry.

Estimated half-life: The N-terminal of the sequence considered is S (Ser).

The estimated half-life is: 1.9 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index: The instability index (II) is computed to be 28.69
This classifies the protein as stable.

Aliphatic index: 60.48

Grand average of hydropathicity (GRAVY): -0.043

Results fro the GbpC protein

Number of amino acids: 583
Molecular weight: 63349.9
Theoretical pI: 5.14

Amino acid composition:

Ala (A)	70	12.0%
Arg (R)	10	1.7%
Asn (N)	41	7.0%
Asp (D)	34	5.8%
Cys (C)	0	0.0%
Gln (Q)	32	5.5%
Glu (E)	44	7.5%
Gly (G)	32	5.5%
His (H)	8	1.4%
Ile (I)	21	3.6%
Leu (L)	27	4.6%
Lys (K)	51	8.7%
Met (M)	6	1.0%
Phe (F)	17	2.9%
Pro (P)	33	5.7%
Ser (S)	35	6.0%
Thr (T)	58	9.9%
Trp (W)	5	0.9%
Tyr (Y)	21	3.6%
Val (V)	38	6.5%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 78
Total number of positively charged residues (Arg + Lys): 61
Atomic composition:

Carbon	C	2775
Hydrogen	H	4344
Nitrogen	N	758
Oxygen	O	927
Sulfur	S	6

Formula: C2775 H4344 N758 O927 S6
Total number of atoms: 8810
Extinction coefficients: Conditions: 6.0 M guanidium hydrochloride
0.02 M phosphate buffer
pH 6.5

Extinction coefficients are in units of M -1 cm -1 .

	276 nm	278 nm	279 nm	280 nm	282 nm
Ext. coefficient	57450	57400	56545	55330	53200
Abs 0.1% (=1 g/l)	0.907	0.906	0.893	0.873	0.840

Estimated half-life: The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index: The instability index (II) is computed to be 25.67

This classifies the protein as stable.
Aliphatic index: 63.02
Grand average of hydropathicity (GRAVY): -0.724

SAPS - Statistical Analysis of Protein Sequences

```
CHARGE DISTRIBUTIONAL ANALYSIS of CSP
        1 00000000+0 00+0000000 +

Positive charge clusters (cmin = 11/30 or 15/45 or 19/60):  none
Negative charge clusters:  not evaluated (frequency of - < 5%, too low)
Mixed charge clusters (cmin = 11/30 or 15/45 or 19/60):  none

CHARGE DISTRIBUTIONAL ANALYSIS of GbpC
         1  0+0+00+000 0000000000 000000--00 00000-0000 000-0000-0 000-+-0000
        61  0000-00000 0000000000 0+-0--0+00 000-00-00- 0-00+0000- 00-0-0+000
       121  00000000-0 0+0+0-000+ 0-000+-0-+ 000-0+-0-0 0+000-00++ -00000000+
       181  0-+0000000 0-0-0+000- 0000000+-0 +0+000--00 00000-0000 0-0000000-
       241  0+--000+0+ 00-000000- 00000+00-+ +00+0+0000 0000000-00 0000000-00
       301  +000000000 000+0-+000 0000000--0 00-0-00000 0000000000 000+00-00-
       361  +0000-0-00 +000000-00 00-0000+-0 00+0000000 0-00-00-00 00-000+000
       421  0000000000 +0-0000000 00-0000000 0000000000 0+-000+000 0-+0-0++00
       481  0000+0000- 0+0--00000 00000--000 -+0+00--00 00000+0000 +00++0-0+0
       541  +-+-000000 00000+0000 0000000000 00000000++ +-0

Positive charge clusters (cmin =  9/30 or 12/45 or 15/60):  none
Negative charge clusters (cmin = 11/30 or 15/45 or 18/60):  none
Mixed charge clusters (cmin = 16/30 or 22/45 or 27/60):  none
```

The following is a pairwise alignment of CSP and antigen C as performed be the NCBI blast algorithm

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.6 [Apr-09-2003]

Matrix BLOSUM62 gap open: 11 gap extension: 1
x_dropoff: 50 expect: 10.00 wordsize: 3 Filter Align

Sequence 1 lcl|seq_1 **Length** 583
Sequence 2 lcl|seq_2 **Length** 21
No significant similarity was found

From the above results, it can be seen that the alignment was not statistically significant to be regarded a match. Therefore a multiple alignment was performed to represent the lack of sufficient amino acid similarity.

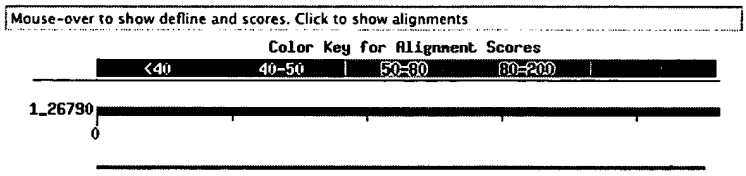
CLUSTALW Result

GenomeNet CLUSTALW Server (Kyoto Center) on Fri Sep 12 02:35:26 JST 2003

```
CLUSTAL W (1.81) Multiple Sequence Alignments

Sequence type explicitly set to Protein
Sequence format is Pearson
Sequence 1: CSP              21 aa
Sequence 2: GbpC            583 aa
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 23.8095
Sequences (2:2) Aligned. Score: 100
Guide tree        file created:   [clustalw.dnd]
Start of Multiple Alignment
There are 1 groups
Aligning...
Group 1:                    Delayed
Sequence:2     Score:119
Alignment Score 0
CLUSTAL-Alignment file created  [clustalw.aln]
CLUSTAL W (1.81) multiple sequence alignment


CSP             ------------------------------------------------------------
GbpC            MKSKTAKITLLSSLALAAFGATNVFADEASTQLNSDTVAAPTADTQASEPAATEKEQSPV

CSP             ------------------------------------------------------------
GbpC            VAVVESHTQGNTTTTTSQVTSKELEDAKANANQEGLEVTETEAQKQPSVEAADADNKAQA

CSP             ------------------------------------------------------------
GbpC            QTINTAVADYQKAKAEFPQKQEQYNKDFEKYQSDVKEYEAQKAAYEQYKKEVAQGLASGR

CSP             ------------------------------------------------------------
GbpC            VEKAQGLVFINEPEAKLSIEGVNQYLTKEARQKHATEDILQQYNTDNYTASDFTQANPYD

CSP             -------------------------------------------------SGSLSTFFR---
GbpC            PKEDTWFKMKVGDQISVTYDNIVNSKYNDKKISKVKINYTLNSSTNNEGSALVNLFHDPT
                                                                  ..:* .:*:

CSP             --LFNRSFTQALGK----------------------------------------------
GbpC            KTIFIGAQTSNAGRNDKISVTMQIIFYDENGNEIDLSGNNAIMSLSSLNHWTTKYGDHVE
                  :*  : *.  *:

CSP             ------------------------------------------------------------
GbpC            KVNLGDNEFVKIPGSSVDLHGNEIYSAKDNQYKANGATFNGDGADGWDAVNADGTPRAAT

CSP             ------------------------------------------------------------
GbpC            AYYGAGAMTYKGEPFTFTVGGNDQNLPTTIWFATNSAVAVPKDPGAKPTPPEKPELKKPT

CSP             ------------------------------------------------------------
GbpC            VTWHKNLVVETKTEEVPPVTPPTTPDEPTPEKPKTPEDPQSPVVAKSVSFRTARKGEMRV

CSP             ----------------------------------------
GbpC            RERDYQPTLPHAGAAKQNGLATLGAISTAFAAATLIAARKKEN

(CSP:0.38095,GbpC:0.38095);
```

BlastP Analysis of the CSP peptide against the whole S. Mutans UA159 Protein Database

Distribution of 1 Blast Hits on the Query Sequence




```
>ref|NP_722220.1| competence stimulating peptide, precursor [Streptococcus mutans
UA159]
Length = 46
Score = 92.0 bits (227), Expect = 3e-21
Identities = 46/46 (100%), Positives = 46/46 (100%)

Query: 1  MKKTLSLKNDFKEIKTDELEIIIGGSGSLSTFFRLFNRSFTQALGK 46
          MKKTLSLKNDFKEIKTDELEIIIGGSGSLSTFFRLFNRSFTQALGK
Sbjct: 1  MKKTLSLKNDFKEIKTDELEIIIGGSGSLSTFFRLFNRSFTQALGK 46


Database: Completed Streptococcus mutans UA159
Posted date:  Jun 2, 2004  2:29 AM
Number of letters in database: 579,702
Number of sequences in database:  1960

Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Hits to DB: 13,265
Number of Sequences: 1960
Number of extensions: 342
Number of successful extensions: 4
Number of sequences better than 1.0e-02: 0
length of query: 46
length of database: 577,947
effective HSP length: 19
effective length of query: 27
effective length of database: 540,859
```

Interpretation

The above analysis is used to show that the CSP protein is found only once in the completed *S. mutans* protein database provided by NCBI (Accession number: NC_004350). The above analysis uses a BLAST algorithm (Basic local alignment search tool) where the query sequence (CSP) is broken up into small sub sequences (called word sizes) and aligned against the full *S. mutans* protein database. Specific details of the algorithms parameters are included above (from the word Matrix onwards). Once an alignment is found, the sub sequence is then extended and scored accordingly to obtain an optimal score. This score is represented by the bit score reported, which is then used to calculate the corresponding E-value. These values are used to represent the

statistical significance of the match found against the database searched. The lower the e-value reported the more significant the match. Also, the amount of amino acids, which match the query sequence, is also reported by the percentage identity value. In this case, there is an exact 100% identity founded between CSP and the CSP protein found in the *S. mutans* database.

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Cloning and Sequence Analysis of the *gbpC* Gene Encoding a Novel Glucan-Binding Protein of *Streptococcus mutans*

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We have isolated dextran-aggregation-negative mutants of *Streptococcus mutans* following random mutagenesis with plasmid pVA891 clone banks. A chromosomal region responsible for this phenotype was characterized in one of the mutants. A 2.2-kb fragment from the region was cloned in *Escherichia coli* and sequenced. A gene specifying a putative protein of 583 amino acid residues with a calculated molecular weight of 63,478 was identified. The amino acid sequence deduced from the gene exhibited no similarity to the previously identified *S. mutans* 74-kDa glucan-binding protein or to glucan-binding domains of glucosyltransferases but exhibited similarity to surface protein antigen (Spa)-family proteins from streptococci. Extract from an *E. coli* clone of the gene exhibited glucan-binding activity. Therefore, the gene encoded a novel glucan-binding protein.

Mutans streptococci have been implicated as the most important microbial agents in dental caries (12), and one of the recognized virulence properties of these organisms is their ability to adhere to tooth surfaces in the presence of dietary sucrose. The roles and mechanisms of glucosyltransferases (GTFs) in adhesion to a variety of surfaces have been investigated by genetic approaches, and many of the genes specifying GTFs have been cloned from streptococci during the past decade (9). These organisms also produce multiple glucan-binding proteins (GBPs) (13, 15), which presumably promote the adhesion of the organisms. Although an 87-kDa GBP (31) from *Streptococcus sobrinus* and 74-kDa (20) and 59-kDa (27) GBPs from *Streptococcus mutans* have been characterized, until now only one GBP (74-kDa Gbp) has been cloned and sequenced (2).

It has been reported (3, 6) that *S. sobrinus* and *Streptococcus cricetus* exhibit rapid aggregation of cell suspensions upon addition of dextran T2000, while *S. mutans* serotype c, e, and f strains fail to do so. Although some GBPs should be involved in this aggregation, no genes responsible for this phenotype in *S. sobrinus* or *S. cricetus* have been identified so far. Subinhibitory concentrations of some antibiotics were reported to have enhanced the dextran-dependent aggregation of *S. sobrinus* (30). We have found that some of the *S. mutans* serotype c and e strains grown with a subinhibitory concentration of tetracycline exhibited rapid aggregation of the cells upon addition of dextran T2000.

In order to define a protein involved in this dextran-dependent aggregation of *S. mutans* based upon a genetic analysis, we constructed random mutants in a transformable *S. mutans* strain by using pVA891 clone banks and isolated dextran-aggregation-negative mutants of *S. mutans*. A chromosomal region responsible for this phenotype was characterized in one of the mutants, and the corresponding gene was cloned in *Escherichia coli* by a marker rescue method. Nucleotide sequence analysis of the gene suggested that the gene encoded a novel GBP associated with the cell surface, and thus we designate this gene *gbpC*. We describe in this communication the cloning, sequencing, and characterization of the gene.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *S. mutans* 109c (serotype c), its spontaneous colonization-defective strain 109cS, and *E. coli* HB101 and JM109 were maintained and routinely grown as previously described (32). *S. mutans* strains and plasmids used in this study are described in Table 1.

Random mutagenesis of *S. mutans* and screening of the mutants. To randomly mutagenize the *S. mutans* chromosome, pVA891 clone banks were utilized as described previously (24, 32). The clone banks were constructed with *Bam*HI-digested plasmid pVA891 (14) and completely *Sau*3AI-digested chromosomal DNA fragments of *S. mutans* 109cS.

All of the transformants detected on Todd-Hewitt agar plates following transformation of strain 109cS with the clone banks were inoculated into 150 μl of BTR broth (25) containing 0.4% glucose (BTR-G) and 0.18-μg/ml tetracycline (BTR-G/tet), which was then dispensed into 96-well microplates. The microplates were sealed with adhesive seal sheets (MS-30010; Sumitomo Bakelite Co. Ltd., Tokyo) to prevent evaporation of medium and incubated overnight at 37°C without shaking. Cells were suspended by pipetting, and a solution of dextran T2000 (molecular weight, 2,000,000; Sigma, St. Louis, Mo.) was added to each well to a final concentration of 100 μg/ml. Microplates were then subjected to shaking with a micromixer (Taitec EM-36; Koshigaya City, Saitama, Japan) for 5 to 10 min at a low to medium range of shaking, the suspensions were observed on a specially devised light box for aggregation, and nonaggregated clones were initially selected as presumable dextran-dependent aggregation-negative (ddag$^-$) mutants.

For the secondary screening, each of the initially isolated mutants was inoculated into three screw-capped glass tubes each containing 2 ml of BTR-G broth, and the tubes were incubated overnight under three different conditions, i.e., incubated with either 0.18 μg of tetracycline/ml or 4% ethanol or incubated at 42°C. Each overnight culture was divided into two 1-ml portions, and dextran T2000 (100 μg/ml) was added to one of them. After swirling for a few minutes, each pair of tubes was observed for aggregation by the unaided eye. Clones exhibiting no visible differences between the pair of tubes from any three incubations were tentatively identified as the ddag$^-$ mutants and stocked frozen at −80°C.

Southern hybridization analysis of ddag$^-$ mutant chromosomes. Appropriate restriction enzyme-digested chromosomal DNA fragments from ddag$^-$ mutants were separated following agarose gel electrophoresis and transferred to nylon membranes (NytranN; Schleicher and Schuell, Dassel, Germany) as described previously (21, 24). The fragments were analyzed by the enhanced chemiluminescence (ECL) direct nucleic acid labelling and detection system as recommended by the supplier (Amersham Co. Ltd., Tokyo).

Recovery of DNA fragments flanking the pVA891 insertion. Following Southern hybridization analysis of chromosomal DNA of the ddag$^-$ mutants, flanking regions containing plasmid pVA891 were recovered in *E. coli* HB101 as previously described (32). The recovered fragments flanking pVA891 were extensively characterized following restriction endonuclease digestion with enzymes selected on the basis of the Southern blot hybridization patterns of this region of the chromosome.

Nucleotide sequencing and sequence analysis. DNA fragments to be sequenced were subcloned into pBluescript KS+/SK+ (Stratagene) or pKmOZ'18/19 (26). Preparation of sequential deletion clones by using the exonuclease III and mungbean nuclease system (Stratagene) and sequencing with *Taq* cycle sequencing kits (PE Applied Biosystems and Amersham Co. Ltd.) and an automated DNA sequencer (PE Applied Biosystems) were accomplished as described pre-

TABLE 1. *S. mutans* strains and plasmids

Strain or plasmid	Description[a]	Source or reference
S. mutans		
109c	ddag$^+$, a clinically isolated transformable strain	This study
109cS	ddag$^+$, a spontaneous colonization defective mutant of 109c due to the homologous recombination between the *gtfB* and *gtfC* genes	This study
32A02	ddag$^-$, a transformant of 109cS isolated following random mutagenesis	This study
LSMD91	ddag$^+$, a deletion mutant of 109cS; *Eco*RV region depicted in Fig. 2 was deleted from chromosome and replaced with the Em^r gene fragment	This study
LSLA91	ddag$^+$, a deletion mutant of 109cS; *Cla*I-*Bst*YI region depicted in Fig. 2 was deleted from chromosome and replaced with the Em^r gene fragment	This study
LSM191	ddag$^-$, a deletion mutant of 109cS; *Xba*I-*Pst*I region depicted in Fig. 2 was deleted from chromosome and replaced with the Em^r gene fragment	This study
LSMF91	ddag$^-$, a deletion mutant of 109cS; *Hin*dIII-*Bst*YI region depicted in Fig. 2 was deleted from chromosome and replaced with the Em^r gene fragment	This study
SM591	ddag$^-$, an insertion mutant of 109cS; the Em^r gene and plasmid pKmOZ'19 was inserted into chromosome as depicted in Fig. 2	This study
LSLL91	ddag$^+$, a deletion mutant of 109cS; *Xba*I-*Ase*I region depicted in Fig. 3B was replaced with the Em^r gene in the same direction with the *gbpC* gene	This study
LSLM91	ddag$^-$, the same as LSLL91 except that the orientation of the Em^r gene was reverse	This study
Plasmid		
pVA891	A marker rescue plasmid containing the Em^r gene used to randomly mutagenize *S. mutans* chromosome	14
pBluescript KS+/SK+	Phagemid cloning vector	Stratagene
pKmOZ'18/19	Km^r-pUC type vector	26
pGEM3zf+	Used as T7 promoter vector in this study	Promega
pGP1-2	A plasmid carrying the T7 RNA polymerase gene	28
pSLC1	The *gbpC* gene was subcloned into pGEM3zf in the same direction as the T7 promoter	This study
pSLU7	5' region of the *gbpC* gene was removed from pSLC1	This study

[a] ddag$^+$, dextran-dependent aggregation positive; Km^r, kanamycin resistance.

viously (32). The nucleotide sequences of both strands of the 2.2-kb *Eco*RV-*Cla*I fragment were determined. Sequence analysis was carried out with the DNASIS-Mac program (Hitachi Software Engineering). The international DNA databases (EMBL, GenBank, and DDBJ) were searched for similar amino acid sequences by using the FASTA program.

Construction of deletion and insertion mutants in *S. mutans*. Deletion and insertion mutants were respectively constructed by allelic exchange and by integration of plasmids containing an internal fragment of the target gene as described previously (32). The predicted deletion of the fragment from or insertion of the plasmid into the chromosome of *S. mutans* was confirmed by Southern hybridization analysis.

Expression of the *gbpC* gene with a T7 RNA polymerase/promoter system. The *gbpC* gene was subcloned into plasmid pGEM3zf (Promega, Madison, Wis.) in the opposite direction relative to the *lac* promoter (in the same direction as the T7 promoter). The resultant plasmid, pSLC1, was then introduced into *E. coli* JM109 harboring the plasmid pGP1-2 coding for the T7 RNA polymerase and expressed according to the previously described procedure (28). As a negative control, about 80% of the 5' region (*Eco*RV-*Bam*HI) of the *gbpC* gene was removed from pSLC1, and the resultant plasmid, pSLU7, was also introduced into the same host. The cells were harvested, washed once with 10 mM potassium phosphate buffer (pH 6.5), and resuspended in 2 ml of the same buffer.

Preparation of crude extracts. *E. coli* cells were grown and suspended as described above. *S. mutans* cells were grown overnight at 37°C in 10 ml of the BTR-G medium, and the cells were collected, washed once, and resuspended in the same buffer at a concentration of 0.2 g (wet weight)/ml. Both *E. coli* and *S. mutans* suspensions were subjected to mechanical disruption with glass beads in a B. Braun MSK cell homogenizer for 5 min and 15 min, respectively in the cold room (4°C). After the removal of the glass beads and cell debris by low-speed centrifugation, the extract was microcentrifuged at 12,000 rpm (10,000 × *g*) for 15 min, and the supernatant was stored at −20°C as a crude extract until use. A portion of the extract was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, and an aliquot was used for the glucan-binding assays.

Glucan-binding assay. Glucan-binding assays were carried out by use of biotin-dextran (molecular weight, 70,000; Sigma) as described previously (11) except for the following modifications. For this assay, the wells of enzyme-linked immunosorbent assay (ELISA) plates (Iwaki Glass, Funabashi, Japan) were coated with 50 μl of *E. coli* crude extracts (0.7 mg of protein per ml) for 18 h at 4°C. *o*-Phenylenediamine dihydrochloride was used as a substrate of the color detection solution, and the resulting A_{490} after incubation with the detection solution for 1.5 min was measured in a microplate reader (Bio-Rad).

SDS-PAGE. A portion of the extract was mixed with the SDS sample buffer and frozen until electrophoretic analysis was performed using the gel system of Laemmli with 7% acrylamide gels. Samples were boiled for 3 min immediately before being applied to the gels. The molecular mass of the protein encoded by the *gbpC* gene was determined following electrophoresis and staining of the gels with 0.1% Coomassie brilliant blue R.

Western blot analysis. Western blot analysis was carried out as previously described but with a slight modification (29). Following SDS-PAGE, the separated proteins were transferred to a supported nitrocellulose membrane, Hybond-C super (Amersham), by a semi-dry electrotransfer apparatus (Model AE6675; ATTO Corp., Tokyo). The nitrocellulose membrane was preincubated with Tris-buffered saline containing 5% bovine serum albumin for 1 h at 37°C to block nonspecific protein binding. The primary antibodies were generated with antiserum either against the *S. mutans* surface protein antigen (Spa) PAc (protein antigen of serotype C [16]) or against the *S. mutans* 59-kDa GBP, GBP59 (27) (kindly supplied by T. Koga, Kyushu University and D. J. Smith, Forsyth Dental Center, respectively), and the secondary antibody was goat immunoglobulin G conjugated to horseradish peroxidase (Organon Teknica Corp., West Chester, Pa.). The ECL Western blotting detection reagent (Amersham) was employed for the detection.

Nucleotide sequence accession number. The *gbpC* nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number D85031.

RESULTS

Dextran-dependent aggregation of *S. mutans* cells. We have found that some *S. mutans* serotype c and e strains grown with a subinhibitory concentration of tetracycline exhibit rapid aggregation of cells upon addition of 100 μg of dextran T2000/ml, although it has been reported that *S. mutans* serotype c, e, and f strains do not aggregate in the presence of dextran T2000 (3, 6). Moreover, similar aggregation could be observed upon addition of dextran into cultures grown under a variety of conditions, including growth at 42°C incubation or growth with 4% ethanol, 80 μg of nalidixic acid per ml, 400 μg of canavanine (nonmetabolizable amino acid analog) per ml, 62.5 μg of spec-

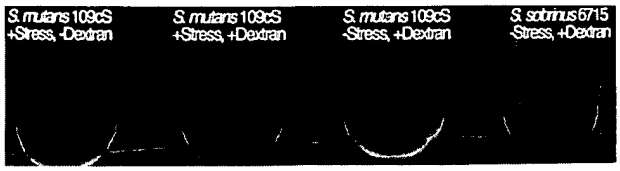

FIG. 1. Dextran-dependent aggregation of *S. sobrinus* 6715 and *S. mutans* 109cS with (+stress) or without (−stress) 0.18 μg of tetracycline/ml.

tinomycin per ml, or 1% ammonium sulfate (data not shown). Most of the reagents added to the medium are known to induce a stress reaction for a variety of cells. Therefore, we tentatively defined these growth conditions as stress conditions. Figure 1 shows the appearance of dextran-dependent aggregated cells of *S. sobrinus* 6715 and *S. mutans* 109cS grown in BTR-G broth (the composition of which was modified in our lab [25]) with (+stress) or without (−stress) 0.18 μg of tetracycline/ml, and aggregation was never observed without dextran T2000. The aggregation of *S. mutans* 109cS was not as strong as that of *S. sobrinus* 6715, as shown in Fig. 1, but it was distinctively visible. The aggregation was competitively inhibited by low-molecular-weight glucan (Dextran T10) and completely inhibited by addition of 1 mM EDTA to the culture. The aggregation was considerably dependent on the growth medium and could not be observed or was difficult to detect with brain heart infusion broth or Todd Hewitt broth. The mechanisms of this dependency on the medium are not known at present. All of the factors described above may have concealed *S. mutans* dextran-dependent aggregation and suggest that aggregation may be controlled by several factors.

Isolation and characterization of dextran-dependent aggregation-negative mutants of *S. mutans*. Construction of a marker rescue plasmid clone bank and random mutagenesis of the *S. mutans* 109cS chromosome were carried out as described in Materials and Methods. Approximately 7,000 colonies were totally screened for the initial step with 10 transformations (about 700 transformants were obtained per transformation procedure), and 69 possible ddag$^-$ mutants were selected. These mutants were then secondarily screened as described in Materials and Methods, and 44 ddag$^-$ mutants were tentatively identified.

*Eco*RI- and *Hin*dIII-digested chromosomal DNA fragments from the 44 ddag$^-$ mutants were analyzed by probing the Southern hybridization patterns with labeled plasmid pVA891 (data not shown). Since a unique *Eco*RI site of pVA891 should be located near the middle of the plasmid when integrated, two bands should be detected in the lanes to which *Eco*RI-digested chromosomal DNAs from the mutants were applied. Meanwhile, only one band is usually detected following the *Hin*dIII digestion, because the unique *Hin*dIII site on pVA891 should be at one end of the integrated pVA891. When the hybridization patterns of the chromosomal DNA from the 44 ddag$^-$ mutants digested with these two enzymes were compared, all of them were different except for those from three mutants. Further comparison of the patterns of *Pst*I- and *Xba*I-digested chromosomal DNAs from the mutants suggested that plasmid pVA891 integrated to the same site on the chromosome in these three mutants. Therefore, one of the mutants, 32A02, was further characterized.

To confirm the genetic linkage of the pVA891 insertion with an alteration of the phenotype in the mutant 32A02, chromosomal DNA from 32A02 was transformed into parental strain 109cS. Several of the transformants examined were all negative in dextran-dependent aggregation. Southern hybridization analysis (data not shown) of the chromosomal DNA of 32A02 and 109cS suggested that a deletion may have occurred in a region flanking the pVA891 on the 32A02 chromosome. In order to recover a deleted region in 32A02, additional flanking regions were obtained from the chromosome of 109cS by chromosomal walking from both ends of the deleted region by using the marker rescue method.

Several deletion and insertion mutants were constructed with the recovered fragments, as described in Materials and Methods. Restriction maps of the fragments used to construct those mutants are indicated with the corresponding chromosomal regions of mutant 32A02 and its parental strain 109cS in Fig. 2. These results suggested that a gene region responsible for dextran-dependent aggregation encompasses a 1.3-kb *Pst*I-*Hin*dIII fragment within the 2.2-kb *Eco*RV-*Cla*I fragment indicated in Fig. 2.

Nucleotide and deduced amino acid sequences of the *gbpC* gene. The nucleotide sequence of the 2.2-kb *Eco*RV-*Cla*I fragment was determined as described in Materials and Methods. The 2,195-bp nucleotide sequence shown in Fig. 3A begins at an *Eco*RV site and ends at a *Cla*I site. The 1,752-bp open reading frame (ORF) encompasses residues 241 to 1992, beginning with an ATG and terminating with a TAG codon. This ORF would encode a 583-amino-acid protein with a calculated molecular weight of 63,478. A potential ribosome-binding site (AGGA) could be identified 8 bp upstream from the ATG initiation codon of the *gbpC* gene. In addition, a promoter-like sequence (TTGAAA-N_{17}-TATAAT), which resembles the *E. coli* promoter consensus sequence, exists between residue 168 and 196, situated 45 bp upstream from the initiation codon of the *gbpC* gene. An inverted repeat sequence characteristic of transcription terminators could also be detected between residues 2072 and 2112, situated 79 bp downstream from the termination codon of the *gbpC* gene. The formation of a putative stem-loop structure in this region of the mRNA corresponds to a free energy change of −115.1 kJ/mol. These results suggest that the *gbpC* gene from *S. mutans* is monocistronic. Since a restriction enzyme cleavage site (*Ase*I) was found immediately upstream from the Shine-Dalgarno sequence of the *gbpC* gene (Fig. 3A), we constructed two mutants of *S. mutans*, LSLL91 and LSLM91, in which the putative promoter regions upstream from the *Ase*I site were removed from the chromosome following replacement with the erythromycin resistance gene, Em^r (see restriction maps in Fig. 3B). These two mutants were isogenic but the direction of the Em^r genes inserted into the chromosome were opposite to each other. LSLL91 exhibited dextran-dependent aggregation similar to the parental strain, even though the *gbpC* gene was not expressed from its own physiological promoter but was apparently expressed by read-through transcription from the Em^r gene, while LSLM91 was ddag$^-$. These results suggest that regulation of the dextran-dependent aggregation of *S. mutans* may be controlled not only by expression of the *gbpC* gene but also by other unknown factors as will be discussed below.

The *gbpC* gene product contained several features generally found in surface proteins of gram-positive bacteria. A Kyte and Doolittle hydrophobicity plot (data not shown) of the deduced protein indicated two apparent hydrophobic stretches at its amino and carboxy termini, and the average value of the whole sequence was calculated to be −0.72. The amino terminus codes a signal peptide-like sequence, although it is not as typical as other streptococcal signal peptides relative to the high content of basic amino acids in the latter. This putative signal peptide may be 39 amino acids long, because of its potential signal peptidase site, and the calculated molecular mass of the putative mature protein should be 59 kDa, which

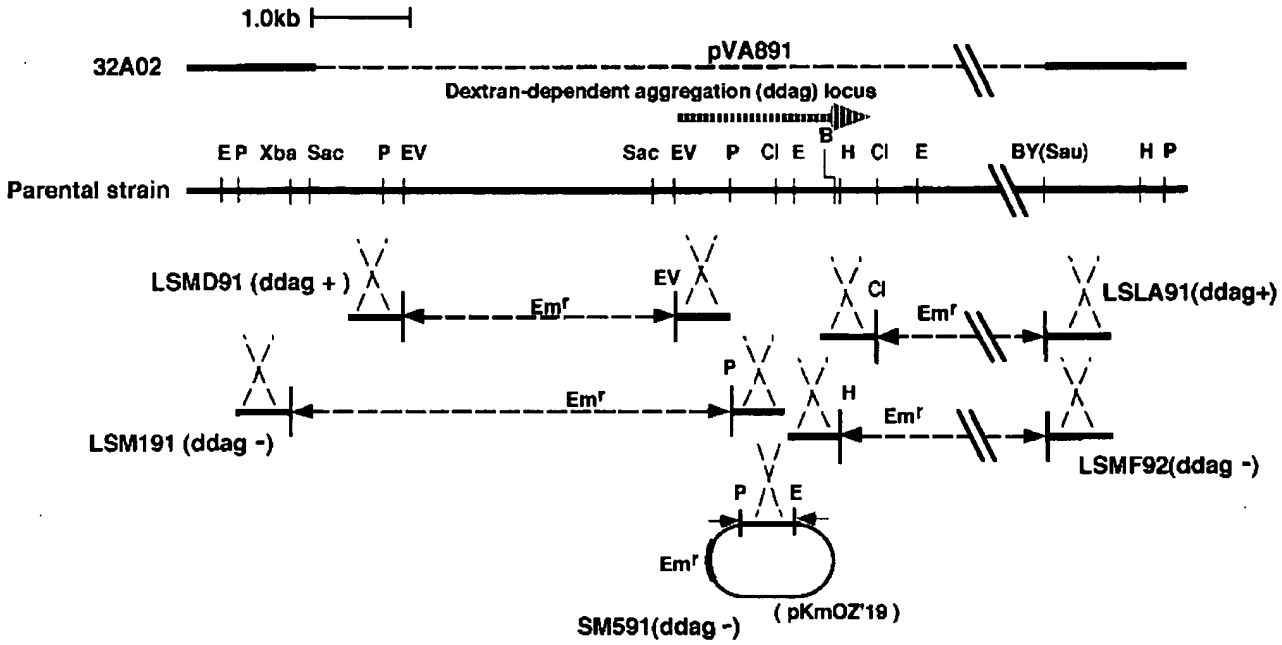

FIG. 2. Restriction map of chromosomal DNA in the mutant 32A02 and parental strain 109cS and the fragments used for construction of deletion and insertion mutants to determine the region responsible for the ddag$^-$ phenotype. In the deletion mutants, the fragments depicted by broken arrows were deleted and replaced by the Em^r gene. Following transformation of 109cS with the linearized plasmids, individual mutants were constructed by double crossover recombination mediated through the homologous regions (depicted by thick lines) which have been subcloned into these plasmids together with the Em^r gene. To construct an insertion mutant, the fragment depicted by the opposing arrows was subcloned into pKmOZ'19 together with the Em^r gene, and the resultant plasmid was then utilized to transform 109cS. The plasmid was integrated into the chromosome by a single recombination event mediated through the homologous fragment. Abbreviations: ddag+, dextran-dependent aggregation positive; B, *Bam*HI; BY, *Bst*YI; Cl, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; P, *Pst*I; Sac, *Sac*I; Sau, *Sau*3AI; Xba, *Xba*I.

is interestingly similar to that of the GBPs recently purified independently by two groups from *S. mutans* (27) and *S. sobrinus* 6715 (13). A consensus sequence for gram-positive cocci surface proteins (L-P-x-T-G-[STGAVDE]) was found at residue 549 of the protein, and the carboxy-terminal sequence (-R-K-K-E-N) was very similar to the membrane anchor region of other surface proteins from gram-positive cocci (8). A possible cell wall-associated region was also found between amino acid residues 497 and 521 and contained 11 proline residues distributed regularly.

Another feature of the overall amino acid composition of the putative mature GbpC protein was high alanine (10.8%) and proline (6.1%) contents, which were similar to those (12.1 and 4.9%, respectively) of the 58- to 60-kDa glucan-binding lectin (GBL) recently isolated from *S. sobrinus* by Ma et al. (13).

The international DNA databases were searched for similar amino acid sequences with the amino acid sequence deduced from the *gbpC* gene. Similar sequences were found in a Spa family of proteins from streptococci, e.g., SpaP, PAc, SpaA, and SSP5, although the molecular sizes of these proteins were obviously different from that of the GbpC protein. The extent of similarities of the corresponding regions between the query sequence and those target sequences ranged from 20.5 to 28.5%. The PAc sequence (18) offered the highest initn score by the FASTA search, while the SSP5 sequence (4) yielded the highest optimized score. The GbpC protein has no similarity to the 74-kDa Gbp of *S. mutans* or the carboxy-terminal repeating domains involved in glucan binding of glucosyltransfereses from mutans streptococci. The sequences of the GbpC protein and the PAc protein of *S. mutans* (serotype c) were partially aligned and are shown in Fig. 4. Although the corresponding region of the PAc protein contains the latter half of the second alanine-rich repeat and the third alanine-rich repeat, such a repeating unit was not detected in the GbpC protein by homology dot matrix.

Expression of the *gbpC* gene in *E. coli* and glucan-binding assays. Since the intact *gbpC* gene could not be subcloned into high-copy-number plasmids, e.g., pBluescript KS+/SK+, in the same direction as the *lac* promoter, a T7 RNA polymerase/promoter system was employed to overexpress the *gbpC* gene in *E. coli* as described in Materials and Methods. When crude extracts from the *E. coli gbpC* clone (pSLC1/pGP1-2) and the control strain (pSLU7/pGP1-2) were examined for glucan binding, it was obvious that the GbpC protein bound glucans, as shown in Fig. 5.

Western blot analysis. Crude extracts from respective strains were subjected to SDS-PAGE. A Coomassie blue-stained gel and a Western blot with antiserum for the PAc protein are presented in Fig. 6. Positive bands with molecular masses of 70, 73, 76, 81, and 95 kDa were detected in lane 3 of the Western blot, which contained the extract of the *E. coli gbpC* clone (pSLC1/pGP1-2) (Fig. 6 [left]); corresponding bands were also detected with the same sample in the Coomassie blue-stained gel. Meanwhile, no positive bands were detected in the control lane (lane 2, pSLU7/pGP1-2) of the Western blot. This means that anti-PAc serum cross-reacted with the GbpC protein. Positive bands detected in a higher-molecular-weight region with an *S. mutans* extract as a positive control (Fig. 6, lane 1) likely represent the PAc protein and its degradation products. However, a positive band corresponding to GbpC protein in the same lane was not detected probably because of low cross-reactivity.

Since our cloned *gbpC* gene encoded a 59-kDa putative

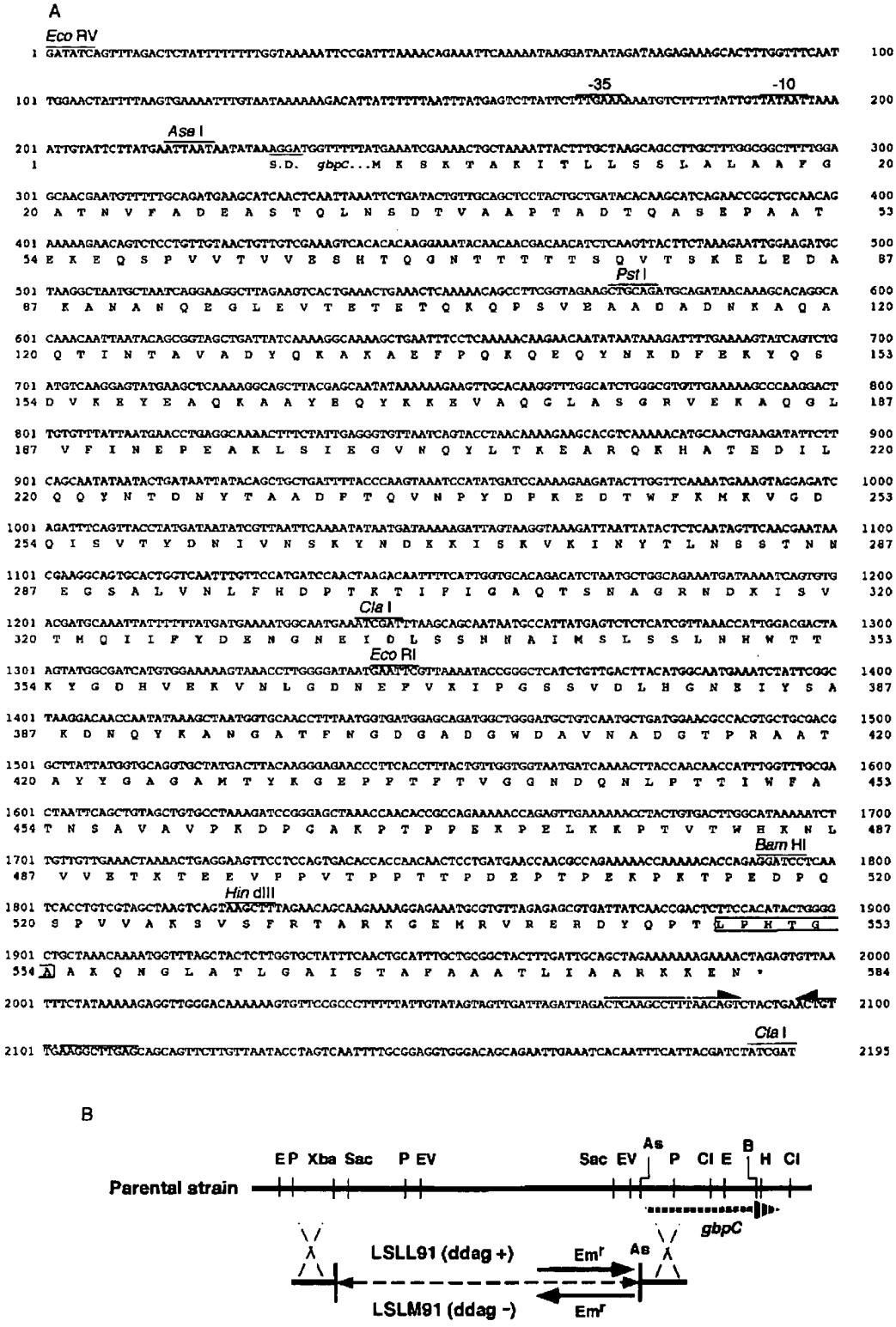

FIG. 3. (A) Nucleotide sequence and deduced amino acid sequence of the *gbpC* gene region. Numbering begins at the *Eco*RV site and the sequence ends at the *Cla*I site 203 bp downstream from the termination codon of the *gbpC* gene. The deduced amino acid sequences specified by the ORFs are presented below the nucleotide sequence. The opposing arrows downstream from the *gbpC* gene denote an inverted repeat. The boxed sequence is a consensus sequence for gram-positive cocci surface proteins (L-P-x-T-G-[STGAVDE]). The nucleotide sequences marked −35 and −10 denote a putative promoter sequence of the *gbpC* gene. A putative ribosome binding site is indicated by S.D. (Shine-Dalgarno). (B) Construction of promoter-deletion mutants. Putative promoter regions upstream from the *Ase*I site were removed from their chromosome by replacement with the Em^r gene oriented in both directions. As, *Ase*I. Other abbreviations are the same as those defined in the legend for Fig. 2.

```
                                10        20        30        40
GbpC                    MKSKTAKITLLSSLALAAFGATNVFADEASTQLNSDTVAAPTADTQ
                                                                  
PAc     MANAANEADYQAKLTAYQTELARVQKANADAKATYEAAVAANNAKNAALTAENTAIKQRNEN
                310       320       330       340       350       360

          50        60        70        80        90       100
GbpC    ASEP-AATEKEQSPVVTVVESHTQGNTTTTTSQVTS--KELEDAKANANQEGLEVTETET
                                                                  
PAc     AKATYEAALKQYEADLAAVKKANAANEADYQAKLTAYQTELARVQKANADAKAAYEAAVA
                370       380       390       400       410       420

          110       120       130       140       150       160
GbpC    QKQPSVEAADADNKAQAQTINTAVADYQKAKAEFPQKQEQYNKDFEKYQSDVKEYEAQKA
                                                                  
PAc     ANNAANAALTAENTAIKKRNADAKADYEAKLAKYQADLARYQKDLADYPVKLKAYEDEQT
                430       440       450       460       470       480

          170       180       190       200       210       220
GbpC    AYEQYKKEVA-QGLASGRVE--KAQGLVFINEPKAKLSIEGVNQYLTKEARQKHATEDIL
                                                                  
PAc     SIKAALAELERHKNEDGNLTEPSAQNLVYDLEPNANLSLTTDGKFLKASAVDDAFSKSTS
                490       500       510       520       530       540
```

FIG. 4. Comparison of the GbpC protein and a portion of the PAc protein of *S. mutans*. Computer-generated alignment of the GbpC and PAc proteins allowing gaps (hyphens) is shown. Identical and similar amino acid residues are marked respectively as colons and dots between the two sequences. PAc is a protein of 1,565 amino acid residues with a cell-associated consensus structure at its carboxy terminus. The numbering of the sequences indicate the actual positions of each amino acid. Aligned with the sequence of the PAc protein is a portion of the surface-exposed domain, where three alanine-rich tandem repeats are present. Brackets on the PAc sequence denote the second and third alanine-rich repeats. v...v, similar region initially detected by the FASTA search.

mature protein with glucan-binding activity, we carried out Western blot analysis with antiserum to the 59-kDa GBP (27), whose antigenicity has been reported to be different from that for the 74-kDa Gbp (2). However, positive bands were detected in the extracts from both the wild type and the mutant of *S. mutans* in which the *gbpC* gene was deleted from its

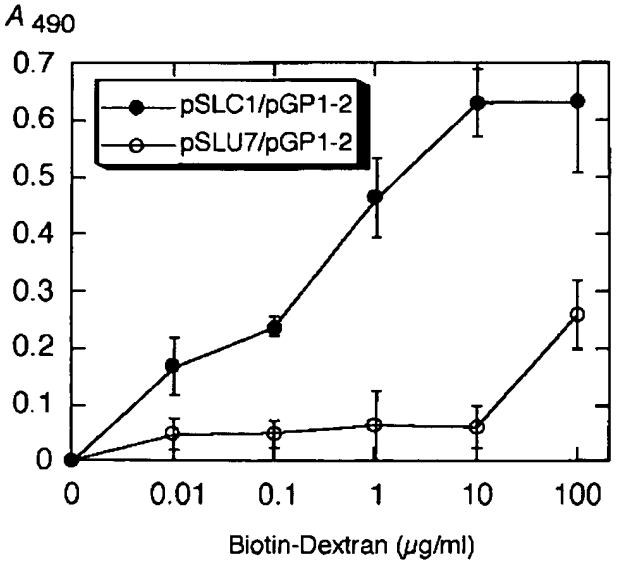

FIG. 5. Comparison of biotin-dextran binding by the GbpC clone and control cell extracts. Wells of ELISA plates were coated with equivalent amounts of protein for each extract and incubated with the indicated concentrations of biotin-dextran, and glucan binding was detected as described in Materials and Methods. The data presented are averages and standard errors of three independent determinations for each sample. Low-molecular-weight glucan (dextran T10) competitively inhibited biotin-dextran binding of the extract from the GbpC clone, and biotin alone did not promote color development (data not shown). Closed circles, extract from *E. coli* JM109 harboring pGP1-2 and pSLC1 (*gbpC* clone); open circles, extract from *E. coli* JM109 harboring pGP1-2 and pSLU7 (control); bars, standard errors.

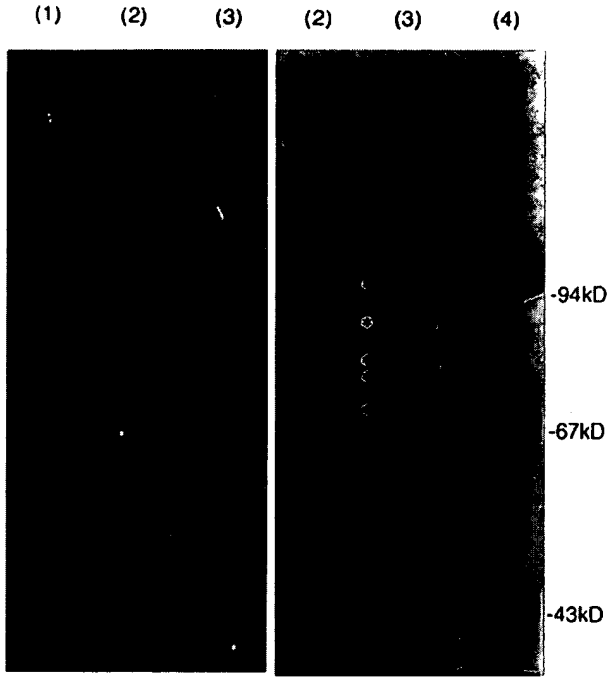

FIG. 6. The result of the Western blot analysis with anti-PAc serum (left) and the Coomassie brilliant blue-stained polyacrylamide gel (right). Cell extracts were prepared as described in Materials and Methods. Equivalent amounts of protein for each extract were applied in the wells. Lane 1, *S. mutans* whole-cell extract; lanes 2, *E. coli* JM109 harboring pGP1-2 and pSLU7 (control); lanes 3, *E. coli* JM109 harboring pGP1-2 and pSLC1 (*gbpC* clone); lane 4, a molecular standard. Asterisks denote bands corresponding to the positive bands in the Western blot.

chromosome and were not detected in the extract from the *E. coli gbpC* clone (data not shown). These results suggest that the protein encoded by the *gbpC* gene is distinct from the 59-kDa GBP (27) and that the GbpC protein is a novel GBP.

DISCUSSION

The dextran-dependent aggregation of 109cS was apparently different from the aggregation by insoluble glucans (33) or by 74-kDa Gbp (5) and is presumed to be independent of the GTFs or GBPs. However, in order to genetically confirm this, we constructed mutants by using strain 109cS in which previously cloned genes specifying GTFs and other proteins were inactivated on their chromosome. These constructs are *gtfB-gtfC-gtfD*, *gtfB-gtfC-gtfD-gbp*, *wapA* (7, 19), and *pac* (17) mutants, and they all exhibited rapid dextran-dependent aggregation (10). Therefore, we anticipated that a previously undescribed gene would be involved in the aggregation and initiated the random mutagenesis strategy.

Mutant 32A02 (Fig. 2) was not a simple pVA891 insertion mutant. The applied mutagenesis method employed entailed the random insertion of pVA891 into the host chromosome by a Campbell-type recombination event mediated between a *Sau*3AI-digested host chromosomal DNA fragment inserted in pVA891 and its chromosomal copy. However, when analyzing the chromosomes of mutants obtained in this experiment as well as those obtained with our previous two other mutagenesis approaches (24, 32), we found relatively large numbers of mutants conferred the target phenotype chromosomal rearrangements of which could not be explained by a single Camp-

bell-type insertion event. Mutant 32A02 (Fig. 2) was one such case, and more than 20 kb (maybe up to 50 kb) of the chromosomal region was deleted from its chromosome following mutagenesis. The constructed partial deletion mutants (Fig. 2), however, revealed that the region responsible for the aggregation was surprisingly small (only 2.2 kb) and is not able to code for a protein with a molecular mass of more than 80 kDa.

The *gbpC* gene coding for the 59-kDa protein possessing glucan-binding activity was identified in this region. Previously, two GBPs in addition to GTFs have been purified from *S. mutans*. The first is a 74-kDa protein coded by the *gbp* gene (2) which has no sequence similarity to the *gbpC* gene. The other is a 59-kDa GBP recently reported by Smith et al. (27), who described its purification and distinct antigenicity to the 74-kDa protein. The results of Western blot analysis revealed that the 59-kDa protein reported by Smith et al. was distinct from our GbpC protein. Therefore, our cloned *gbpC* gene codes for a novel, third GBP, which is very likely a surface protein involved in the glucan-dependent aggregation.

Ma et al. (13) recently reported a 58- to 60-kDa GBL from *S. sobrinus* and suggested that a GBL is defined as a GBP capable of conferring upon a bacterium the ability to be aggregated by α-1,6 glucan. According to this definition, the GbpC protein can be classified as a GBL. Although no information concerning the amino acid sequence of the *S. sobrinus* GBL is available, its molecular weight and amino acid content of proline and alanine are similar to those of the *S. mutans* GbpC protein. Inhibitory effects of chelating agents or dextran T10 on the glucan-dependent aggregation of both organisms were also similar. Taken together with the above information, the GbpC protein may be a counterpart of the *S. sobrinus* GBL.

A FASTA homology search indicated similarity between the GbpC protein and a family of streptococcal surface proteins, and antiserum for PAc cross-reacted with the GbpC protein overexpressed in *E. coli*. Previous reports have stated that the SpaA protein from *S. sobrinus* is a GBP (1, 3, 6). Some common structures which are able to bind glucan may exist on these two proteins because of sequence similarity and antibody cross-reactivity, although the GbpC protein is obviously involved in the dextran-dependent aggregation but the PAc protein is not (see above). The positive bands in Western blots were unexpectedly multiple, and the same-sized protein bands could also be recognized in a Coomassie-stained gel. Even for the smallest band, its size was larger than the calculated molecular weight of the GbpC protein. How these multiple protein products were generated is not known at present. The *gbpC* gene product may be toxic for *E. coli*, since the gene could not be subcloned into high-copy-number plasmids in the same direction as the *lac* promoter. Therefore, *E. coli* cells may have posttranscriptionally modified the GbpC protein in this case in order to detoxify it.

We expected the mutant LSLL91 to exhibit the constitutive dextran-dependent aggregation because the *gbp* gene in this mutant could be transcribed from the Em^r gene promoter. However, like the parental strain, the mutant cells aggregated with dextran only after growth under stress conditions. These results suggest that expression of the *gbpC* gene is essential but probably not sufficient for the dextran-dependent aggregation. The GbpC protein may need to be posttranscriptionally modified or requires other factors for aggregation. We have obtained some data to support this hypothesis. (i) Besides the mutant 32A02, we obtained about 40 of the $ddag^-$ mutants, which have not been characterized in detail. Some of them appeared to retain the intact *gbpC* gene, since chromosomal DNA fragments analyzed by Southern hybridization following digestions with several restriction enzymes were the same sizes as those of parental strain 109cS. (ii) We found that two serotype f strains, OMZ175 and 130f, did not aggregate with dextran T2000. These strains also carried the *gbpC* gene as shown by Southern hybridization analysis. (iii) We have detected a small ORF downstream from the *scrB* gene previously characterized in our labs (22, 23) with strain GS-5. When a mutant was constructed with the ORF inactivated in the chromosome of strain 109cS, this mutant constitutively exhibited the dextran-dependent aggregation under nonstress conditions. This ORF is an example of a possible factor involved in aggregation. However, we do not yet know whether this ORF controls transcription of the *gbpC* gene. We have no data regarding transcriptional regulation for any of the mutants or the parental strain at present. Moreover, the other factors regulating the dextran-dependent aggregation remain to be determined. Such further studies are currently in progress.

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RAPID COMMUNICATION
Biological

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Attenuation of Glucan-binding Protein C Reduces the Cariogenicity of *Streptococcus mutans*: Analysis of Strains Isolated from Human Blood

ABSTRACT

A blood isolate of *Streptococcus mutans* strain TW871 shows relatively low homology with MT8148, a reference oral isolate strain, and lacks the serotype-specific polysaccharide antigen, suggesting that other cell-surface structures correlate with cariogenicity. We compared cariogenicity of TW871 with MT8148 (serotype *c*) and blood isolate TW964 (serotype *f*) in rats. Strain TW871 showed significantly lower cariogenicity than MT8148 or TW964 and expressed significantly lower sucrose-independent cellular adhesion to saliva-coated hydroxyapatite and dextran-binding activity than strain MT8148. Strains TW871 and TW964 showed a defect in the *gbpA* gene by Southern hybridization analysis, while sequencing analysis revealed *gbpC* variation in TW871. These results suggest that variation in GbpC may alter cellular adherence properties and can be correlated with the cariogenicity of *S. mutans* in this strain.

KEY WORDS: *Streptococcus mutans*, bacteremia, infective endocarditis, cariogenicity, glucan-binding protein C.

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INTRODUCTION

Streptococcus mutans has been implicated as a primary etiologic agent of dental caries in humans (Hamada and Slade, 1980). The cell-surface protein antigen (PAc) and 3 types of glucosyltransferases (GTFs) have been investigated for cariogenicity of *S. mutans*. *S. mutans* also synthesizes glucan-binding proteins as cell-surface proteins. Thus far, GbpA (Russell *et al.*, 1985), GbpB (Smith *et al.*, 1994), and GbpC (Sato *et al.*, 1997) have been purified, and the genes *gbpA* (Banas *et al.*, 1990), *gbpB* (Mattos-Graner *et al.*, 2001), and *gbpC* (Sato *et al.*, 1997), encoding GbpA, GbpB, and GbpC, respectively, have been cloned and sequenced. However, their contribution to the cariogenicity of *S. mutans* is still uncertain.

S. mutans is occasionally isolated from the blood of patients with bacteremia and infective endocarditis (Hamada and Slade, 1980). In our previous study, 4 streptococcal strains isolated from human blood were identified as *S. mutans* based on their biological properties and 16S ribosomal RNA sequences. However, DNA-DNA hybridization analysis of strain TW871 also showed a low homology of 76.3% when compared with the reference strain, MT8148. In addition, TW871 has been shown to have lost the serotype-specific polysaccharide antigen on the cell surface, making it serologically untypable (Fujiwara *et al.*, 2001). These findings suggest the possibility that other cell-surface structures correlated with the pathogenicity of dental caries may vary. The purpose of the present study was to examine the caries-inducing activity of these blood isolates in SPF rats and define the association of glucan-binding proteins with the cariogenicity of *S. mutans*.

MATERIALS & METHODS

Bacterial Strains

S. mutans TW871 (serotype, untypable) and TW964 (*f*) were isolated from infective endocarditis patients and used in the present study. *S. mutans* MT8148 (*c*), isolated from the oral cavity of a healthy child, was used as the reference strain. For animal experiments, these 3 strains were made resistant to streptomycin (1500 μg/mL) and termed TW871R, TW964R, and MT8148R, respectively. For a saliva-coated hydroxyapatite adherence assay, we prepared [^{3}H]-labeled cells by culturing them in Brain Heart Infusion (BHI; Difco Laboratories, Detroit, MI, USA) broth containing [^{3}H]-thymidine (0.1 MBq/mL; Moravek Biochemicals Inc., Brea, CA, USA). The isolation of the bacteria from patients was carried out in accordance with the guidelines established by the Japanese Public Health Service and the Osaka University Health Guidelines for Studies Involving Human Subjects.

Caries Induction in Animal Experiments

All animal procedures and protocols were approved by the Animal Experiment Committee of Osaka University Graduate School of Dentistry. The caries-inducing activities were examined with the use of 45 specific pathogen-free (SPF) Sprague-Dawley rats (15 rats *per* group) (CLEA-Japan, Osaka, Japan), and the plaque scores, recovery of the inoculated strains, and caries scores of each rat were evaluated according to the method described previously (Ooshima *et al.*, 1991).

Table. Caries-inducing Activity of *S. mutans* Strains Isolated from Human Blood

Strains (serotype)	Bacterial Recovery CFU x 10^4/mandible (mean ± SE)	Plaque Index (mean ± SE)	Caries Scores (mean ± SE) Smooth	Total
MT8148R (c)	55.7 ± 12.2	1.1 ± 0.1	21.8 ± 2.0	78.1 ± 3.6
TW871R (-)	17.6 ± 8.4[a]	0.5 ± 0.0[b]	9.4 ± 0.9[b]	39.0 ± 2.5[b]
TW964R (f)	96.8 ± 22.9	1.1 ± 0.1	21.8 ± 1.8	75.8 ± 4.5

There were statistically significant differences between the rats infected with MT8148R and the other groups of rats by Fisher's PLSD analysis.
[a] $P < 0.05$.
[b] $P < 0.001$.

Construction of a *gbpC*-defective Mutant

The coding region of *gbpC* of MT8148 was amplified by polymerase chain-reaction (PCR) with AmpliTaq Gold^R polymerase (Applied Biosystems, Foster City, CA, USA), with primers constructed on the basis of the *gbpC* sequence from *S. mutans* strain 109c (Sato *et al.*, 1997), and then cloned into a pGEM^R-T Easy Vector (Promega, Madison, WI, USA) to generate pMM5. The *gbpC* gene fragment from pMM5 was ligated into plasmid pUC19 (Takara, Kyoto, Japan) and cleaved with *Pvu* II to yield pMM7. The open reading frame (ORF) of *gbpC* in pMM7 was cleaved and blunted in the middle, then ligated with a kanamycin-resistant gene (*aphA*; Caillaud *et al.*, 1987) cassette to yield pMM8. After linearization by digestion at the unique *FspI* restriction site, the plasmids were introduced into *S. mutans* MT8148 by the method of Tobian and Macrina (1982).

Anti-GbpC Antiserum

The generated pMM5 was digested with *Nco* I and *Sac* I, and then the *gbpC* gene fragment was ligated into a pET-32a (+) vector (Novagen, Madison, WI, USA). Recombinant GbpC (rGbpC) was expressed with the use of *E. coli* BL21(DE3) (Novagen), the cells were harvested by centrifugation, and rGbpC was extracted with B-PER™ Bacterial Protein Extraction Reagent (Pierce, Rockford, IL, USA). Crude rGbpC was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the target band was excised, then homogenized in saline and mixed with Freund's complete adjuvant (Difco), which was injected 3 times intramuscularly over a 14-day interval into adult white rabbits. Two weeks after the third injection, blood was drawn, and antiserum was collected and stored at –20°C.

Dextran-binding Assay

Dextran-binding activity was evaluated by the method of Lis *et al.* (1995) with biotin-dextran solution (Sigma, St. Louis, MO, USA) and horseradish-peroxidase-conjugated streptavidin.

Sucrose-independent Cellular Adhesion to SHA

An assay for the sucrose-independent adhesion of *S. mutans* to saliva-coated hydroxyapatite (SHA) was performed by the method described by Matsumoto *et al.* (1999) with some modification as follows. We calculated the specific binding level by subtracting the non-specific binding level using saliva-noncoated hydroxyapatite according to the method described by Nakagawa *et al.* (2000).

Southern Hybridization and Western Blot Analyses

Southern hybridization analyses of *gbpA* and *gbpC* genes with *EcoR* I, *Hind* III, or *BamH* I, and Western blot analysis of GbpC with whole-cell lysates of the tested strains were carried out with standard procedures as described previously (Fujiwara *et al.*, 2000).

Sequence of *gbpC* Genes

The sequences of these genes were determined with a DNA Sequencing System (373-18 DNA sequencer, Applied Biosystems) and an ABI PRISM Cycle Sequencing kit.

Statistical Analysis

Intergroup differences of various factors were estimated by a statistical analysis of variance (ANOVA) for factorial models. We used Fisher's protected least-significant difference test to compare individual groups.

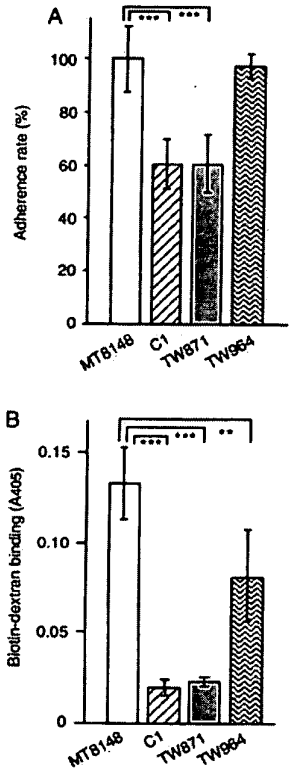

Figure 1. Cellular adhesion and dextran-binding activities of TW strains and MT8148 (mean ± SD; n = 5). **(A)** Sucrose-independent cellular adhesion to saliva-coated hydroxyapatite of MT8148, GbpC-defective mutant (C1), and TW strains. **(B)** Dextran-binding activity of MT8148, C1, and TW strains. There were statistically significant differences between MT8148 and the other strains by Fisher's PLSD analysis. (**$P < 0.01$, ***$P < 0.001$).

RESULTS

The Table shows the caries-inducing activity of the *S. mutans* strains isolated from blood. Strain TW871R showed a significantly lower level of bacterial recovery from the mandible as well as a lower plaque index and caries score than strain MT8148R, while strain TW964R showed a caries-inducing activity similar to that of MT8148R.

Fig. 1A shows the sucrose-independent cellular adhesion of MT8148, TW871, TW964, and C1, a *gbpC*-defective mutant of MT8148. The cellular adhesions of TW871 and C1 were both approximately 60% of MT8148, significantly lower than that of

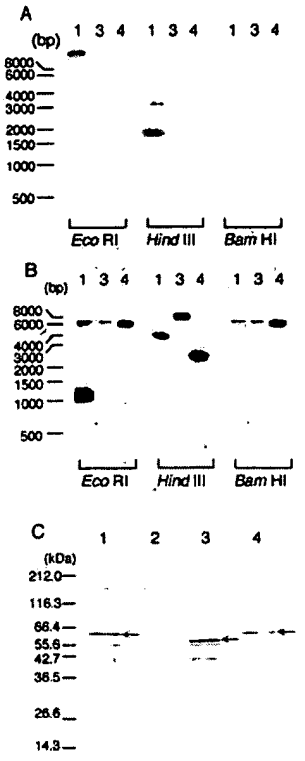

Figure 2. Identification of *gbpA*, *gbpC*, and expressed GbpC. Southern hybridization analyses of *S. mutans gbpA* (**A**) and *gbpC* (**B**), and Western blot analysis of GbpC (**C**), among MT8148 and TW strains. The arrows indicate GbpC of each strain. Lanes: 1, MT8148; 2, C1; 3, TW871; and 4, TW964.

MT8148 ($P < 0.001$ and $P < 0.001$, respectively). On the other hand, TW964 showed adhesion similar to that of strain MT8148, and both were significantly higher than that of TW871 ($P < 0.001$). The dextran-binding assay showed that TW871 possessed significantly lower dextran-binding activity, equivalent to that of the *gbpC*-defective mutant C1 (Fig. 1B). TW964 showed a significantly lower level of dextran-binding activity as compared with MT8148, but was significantly higher than that of TW871 ($P < 0.001$).

Southern hybridization analysis indicated the absence of a *gbpA* homolog in strains TW871 and TW964 (Fig. 2A). However, all of the TW strains showed the *gbpC* gene when they were hybridized with a fragment of *gbpC* (Fig. 2B). The differences in patterns for the TW strains relative to MT8148 for *gbpC* were apparent. Western blot analysis showed that MT8148 and TW964 possessed an approximately 63-kDa protein when reacted with anti-GbpC antiserum (Fig. 2C; lanes 1 and 4). As for TW871, the positive band was estimated to be an approximately 59-kDa protein (Fig. 2C; lane 3).

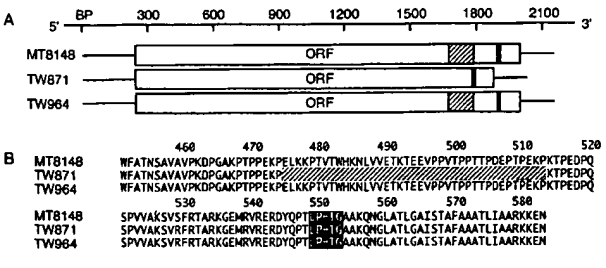

Figure 3. Putative structure of GpbC of TW strains and MT8148. (**A**) Map of putative nucleotide structure of *gbpC* among MT8148 and TW strains. A base pair (BP) scale is illustrated above the map. ▮ Cell wall anchored region. ▨ 39 amino acid deletions seen in TW871 strain. (**B**) C-terminus deduced amino acid alignment of GbpC of MT8148 and TW strains. 460-580 at the top of (B) indicates the serial number of deduced amino acids in MT8148.

Fig. 3A illustrates the putative ORF in *gbpC* among the TW strains, compared with that of MT8148. The ORF sequence in *gbpC* of strains TW964 was shown to be completely identical to that of strain MT8148. On the other hand, the *gbpC* gene in TW871 lacked 117 bp (hydrophilic 39 amino acids) close to the C-terminal membrane-anchoring region (Fig. 3B).

DISCUSSION

The etiology and pathogenesis of *S. mutans* in dental caries are generally studied with strains isolated from the oral cavity. However, the cariogenicity of those isolated from the blood of bacteremia and infective endocarditis patients has not been reported. In the present study, the serologically untypable strain TW871 showed significantly lower levels of cariogenicity. Several properties related to the cariogenicity of *S. mutans*—including sucrose-dependent cellular adhesion, cell hydrophobicity, and GTF activity—were found to be similar to those of strain MT8148 (Fujiwara *et al.*, 2001); therefore, we attempted to examine the sucrose-independent cellular adhesion and dextran-binding activity of the TW strains in detail. Our results suggested the existence of variations or deletions of glucan-binding proteins (Gbps).

Gbps are considered to be involved with dental caries. Anti-GbpA antisera were shown to cause a reduction in the sucrose-dependent adherence of *S. mutans* (Douglas and Russell, 1982), and a *gbpA*-defective mutant was reported to lack the ability to form adherent colonies in the presence of sucrose (Russell *et al.*, 1985). In the present study, a *gbpA* gene defect was found in strains TW871 and TW964. TW871 also showed *gbpC* gene variations, and its cariogenicity in the rat experiment was significantly lower than that of the reference strain, MT8148R. On the other hand, strain TW964 had an intact *gbpC* gene and showed cariogenicity equal to that of strain MT8148R. These results suggest a low possibility that a *gbpA* defect alone may be the cause of the drastically reduced cariogenicity in *S. mutans*. However, the role of GbpA in *S. mutans* cariogenicity should be examined in a *gbpA*-defective mutant, since TW964 is a blood isolate and may possess other unknown variations.

GbpC is regarded as a cell-associated protein with a high homology to PAc (Sato *et al.*, 1997), which has been reported to participate in sucrose-independent SHA adherence and to have a correlation with the cariogenicity of *S. mutans* (Koga *et al.*, 1990). In the present study, the presence of the *gbpC* gene was recognized in all of the isolates by Southern hybridization analyses, whereas variations of it were assumed in strain TW871 from the results of Western blot analysis. Sequence analysis showed that the *gbpC* gene in strain TW871 lacked 117bp. In strain TW871, the SHA adhesion rate was approximately 70% of that of MT8148, and bacterial recovery from the rats was also significantly lower. Furthermore, the dextran-binding activity of TW871 was at an extremely low level, similar to that of the GbpC-defective mutant C1. These results suggest that the conformation change of GbpC in strain TW871 may impair the bacterial attachment mechanism and reduce its caries-inducing activity. In the additional experiment, the mean total caries score (42.5) of the rats infected with C1 was significantly lower than that of MT8148R (56.8), suggesting that GbpC may play an important role in *S. mutans* cariogenicity.

Infective endocarditis is known to be initiated by an invasion of pathogenic bacteria into the bloodstream, whereas the mechanisms of invasion and survival of *S. mutans* in blood have not yet been elucidated. Analysis of serum antibody response in normal human subjects suggests that GbpC exhibits a significantly higher reaction with salivary IgA and also serum IgG than other antigens, including PAc (Chia *et al.*, 2000). Therefore, a variation of GbpC may cause a weak immune response, allowing *S. mutans* to survive in the bloodstream.

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